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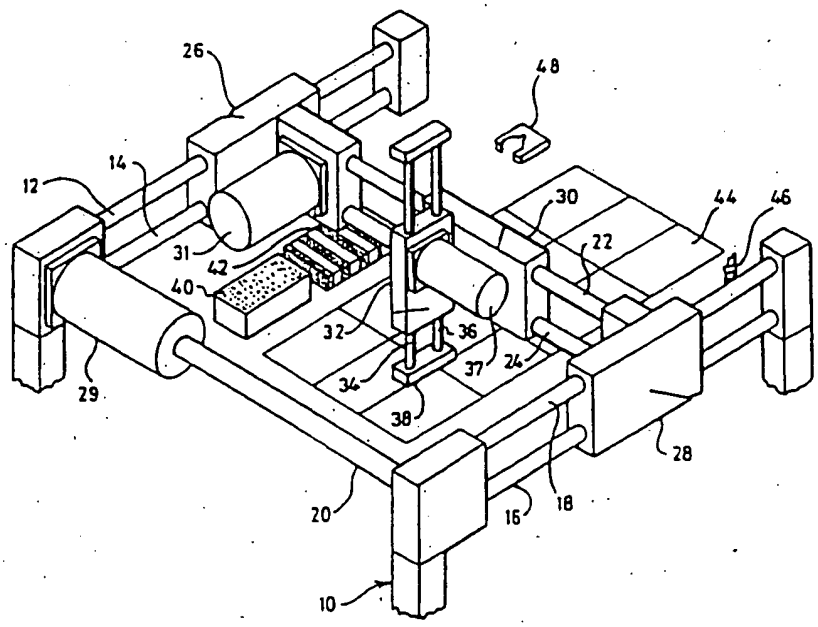
(54) Title: AUTOMATED PREPARATION OF NUCLEIC ACIDS



(57) Abstract

Disclosed is apparatus for preparing a DNA sequence of interest from a complex sample comprising use of magnetic particles in an assay plate, said apparatus comprising: means for moving an assay plate in three-dimensional space; means for supplying liquids to the assay plate; thermal regulation means for controlling the temperature of the assay plate; magnetic separation means for manipulating magnetic particles in the assay plate; and computer control means for controlling and co-ordinating operation of all the above means and an automated method of preparing DNA from a complex sample.

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Title Automated Preparation of Nucleic Acids

Field of the Invention

This invention relates to the automated preparation of nucleic acids and concerns an automated method of nucleic acid preparation and automated apparatus therefor.

Background of the Invention

Currently, a number of large scale DNA sequencing projects are under way. One of the rate-limiting steps in the generation of sequence data is the preparation of high purity DNA templates.

Typically, M13 phage is used to obtain single stranded DNA for sequencing templates. The traditional methods for M13 DNA purification, such as polyethylene glycol (PEG)/phenol procedures (Bankier et al., [1988], in Wu, R. [ed.], Methods Enz. 155, 52-93) allow microgram quantities of template to be produced from millilitre volumes. However, thermally cycled sequencing procedures utilising Taq polymerase (Craxton, [1991], Methods: A Companion to Methods in Enzymology 3, 20-26) only require 200-500ng of template DNA per reaction. Therefore traditional methods produce an excess of template for most purposes, which wastes both time and money.

Some alternative methods of preparing template DNA have been described which employ a microtitre-scale format, and thereby reduce waste. For example, one method uses

specialised filtration steps (Kristensen et al., [1987], Nucleic Acids Research 15, 5507-5516), whilst another (Smith et al., [1990], Journal of DNA Sequencing and Mapping 1, 73-78) requires the use of PEG/sodium dodecyl sulphate (SDS) with multiple centrifugation steps. Thus these alternative techniques are somewhat cumbersome and impractical.

More recently, a protocol has been described (Alderton et al., [1992], Analytical Biochemistry 152, 304-307) which utilises magnetic particles together with PEG-phage aggregation. A somewhat similar method is disclosed in WO90/06045. This outlines a method of preparing DNA using magnetic particles coated with oligonucleotides, which serve as hybridisation probes. The particles and any bound nucleic acids can then be magnetically separated from the rest of the sample.

An improved method of preparing nucleic acids (using magnetic particles), particularly preparing template DNA for sequencing, is described in GB 9212164.9 (co-pending International Application No. PCT/GB93/).

Advances in techniques involving preparation or manipulation of DNA have allowed the introduction of automated apparatus for some processes. For example, automated apparatus for performing the polymerase chain reaction (PCR) and for performing DNA sequencing reactions are known.

Summary of the Invention

In one aspect the invention provides apparatus for preparing a DNA sequence of interest from a complex sample

comprising use of magnetic particles in an assay plate, said apparatus comprising:

means for moving an assay plate in three-dimensional space;
means for supplying liquids to the assay plate;
means for removing liquids from the assay plate;
thermal regulation means for controlling the temperature of the assay plate;
magnetic separation means for manipulating magnetic particles in the assay plate; and
computer control means for controlling and co-ordinating operation of all the above means.

Preferably the assay plate is of a synthetic plastics material which is suitable for use at temperatures above 50°C, (more preferably at temperatures up to 80°C). It is also preferred that the assay plate comprises a microtitre assay plate, typically with 96 wells.

The apparatus of the invention conveniently comprises a robotic head capable of movement in all three dimensions (thus known as an "X, Y, Z robot") with position sensing means, such that the location of the head in three-dimensional space is known relative to a fixed point.

The head is preferably capable of pipetting and dispensing liquids.

Convenient pipetting devices are known (e.g. those manufactured by Hook and Tucker) and may be attached to the robot head by tubing. Aspiration means are typically provided by a vacuum pump (such as that available from Charles Austen) connected to the robot head by valves and

tubing. Typically liquids are dispensed by a plurality of disposable tips, such as well known to those skilled in the art. Ideally, the apparatus comprises a store of disposable sterile tips ready for use and a receptacle for the collection of used tips. The apparatus should preferably include tip-sensing means such that the number of tips located on the robot head is known, as it is possible that tips may not become properly attached to the head.

Any magnetic particles to which are attached an appropriate probe are suitable for use with the apparatus defined above, including those already known in the art. However, it is preferred that the particles used are those described in GB 9212164.9 (co-pending International Patent application No. PCT/GB93/).

The magnetic separation means conveniently comprise two separate workstations. One such workstation may comprise bar magnets of a size and number such that a typical microtitre plate may rest on the magnets such that each row of wells in the plate aligns with a bar magnet. Such a station may be termed the "separation" station, which may be used to separate magnetic particles from mixtures by attracting said particles to the magnets. The other magnetic workstation may comprise bar magnets of a size and number such that two rows of wells may be positioned between each bar magnet, allowing for movement of the plate relative to the magnets, such that the magnets may be situated between different rows of wells on the microtitre plate. This station may be termed the "washing" station. Washing can be effected by adding washing medium to the wells of the microtitre plate and drawing the magnetic particles to one side of the wells.

After a suitable period (for example, 5 seconds), the plate is moved relative to the magnets so that the magnet is on the other side of the wells, thereby dragging the magnetic particles through the washing medium.

In an alternative embodiment, the magnetic workstation(s) may comprise "dot" magnets instead of bar magnets. Preferably an array of dot magnets is employed, each magnet being approximately the diameter of one of the wells of the assay plate. Preferably the dot magnets are rare earth magnets and are arrayed in a matrix 8 x 12 (i.e. that of a typical 96 well microtitre plate) having dimensions 130mm x 85mm, the array being such that each magnet is separated from its nearest neighbours by the same spacing as that between adjacent wells of the microtitre plate (typically about 9mm) such that a rare earth dot magnet may be positioned substantially directly beneath each well of the assay plate.

The thermal regulation means conveniently comprises three heating blocks each set at a constant desired temperature, with the microtitre plate being transferred from one to another to alter the temperature of the plate. However, other arrangements may be envisaged by those skilled in the art. For example, a single heating block with a controlled variable temperature may be employed.

Conveniently the apparatus includes a static baseplate, upon which various accessories may be located (for example, a store of microtitre plates awaiting processing).

The overall operation of the apparatus is controlled by computer means, typically a personal computer. Such a

computer would control, for example, the movement of the robot head and microtitre plates, the pipetting/dispensing of liquids and the temperature of the thermal regulation means, typically via an RS232 computer interface.

In another aspect, the invention provides an automated method of preparing a DNA sequence of interest from a complex sample, comprising: use of automated means for the steps of causing lysis of DNA-containing entities by the action of heat in the presence of a suitable detergent; adding magnetic particles attached to a probe together with hybridisation buffer containing a polymer to cause an increase in the effective nucleic acid concentration; allowing the sequence of interest to hybridise to the complementary probe and then separating the particle/probe complex and any sequences hybridised thereto from the rest of the sample by magnetic attraction.

A suitable detergent is SDS.

Preferably the steps of the method are performed in the wells of a microtitre assay plate.

The DNA-containing entities are generally phages, typically M13.

A suitable polymer is PEG. The use of a polymer to increase the effective concentration of nucleic acid allows smaller volumes of reagents to be used than previously, which in turn enables the method to be performed in a microtitre plate or tray. Similarly, the method is particularly simple and efficient as it employs a target sequence-specific purification step.

One embodiment of apparatus in accordance with the invention and an Example illustrating its use will now be described, by way of illustration, and with reference to the accompanying drawings in which:

Figure 1 is a schematic representation of one embodiment of the apparatus of the invention;

Figure 2 shows the base sequence of a suitable probe which may be used in the method of the invention;

Figure 3 shows a photograph of gel electrophoresis which may be performed on DNA prepared using the method of the invention; and

Figure 4 shows a portion of a sequencing trace which may be obtained using DNA prepared using the method of the invention.

Detailed description of drawings

Figure 1 is a schematic representation of automated apparatus in accordance with the invention, suitable for performing the DNA preparation method of the invention. The apparatus includes a number of standard, commercially available components, which, in the interests of clarity, are not shown in the figure.

The illustrated apparatus comprises an X-Y-Z robot 10, e.g. as produced by Burger Lahr, Aerotech or Linear Technology. Robot 10 comprises a base plate (not shown) supporting a rectilinear framework, including rails 12, 14 and 16, 18 extending in the 'X' direction and rails 20 and

22, 24 extending in the 'Y' direction. Rails 22, 24 are mounted on rails 12, 14 and 16, 18 via respective support blocks 26 and 28, permitting sliding movement of the Y rails in the X direction under the action of drive means 29. A support block 30 is carried on rails 22, 24, arranged for sliding movement thereon in the Y direction under the action of drive means 31. Block 30 has fixed thereto a further support block 32, through which extend a pair of rails 34, 36 arranged for movement relative to block 32 in the Z direction under the action of drive means 37. A robot head 38 is carried at the lower end of rails 34, 36. Robot head 38 is movable relative to the base plate in 3 dimensional space under the control of computer control means (not shown) acting to cause appropriate movement of blocks 26, 28, block 30 and rails 34, 36 by the associated drive means.

The robot head is designed to transport a standard 96 well microtitre plate (not shown) between different locations within the framework. To this end the head has fixed thereto vacuum pads (from Kosma) and associated vacuum control means (not shown) for suitable attachment to a microtitre plate.

The robot head is also designed to perform pipetting and aspirating functions. The apparatus thus includes a pipetting device manufactured by Hook and Tucker (not shown) linked to the robot head by means of suitable tubing (not shown). For aspiration purposes the apparatus also includes a vacuum pump (from Charles Austen) and associated valves and reagent lines (not shown) leading to the robot head.

The base plate supports a number of different components

required for performing the method. In particular the plate supports three heat blocks from Techne, one of which is shown schematically at 40 in the figure. Each heat block is adapted to be heated to a different fixed temperature under the computer control means. The base plate also carries two magnetic separation stations, e.g. from Techne, one of which is illustrated schematically at 42 in the figure. Each magnetic separation station comprises an array of bar magnets arranged in parallel spaced relationship.

One magnetic separation station is designed to retain magnetic beads in the base at the microtitre plate wells while liquid is aspirated off, thus separating the beads. This station comprises an array of eight bar magnets separated by 9mm gaps so as to be located beneath adjacent rows of wells in a microtitre plate.

The other magnetic separation station is designed to perform a washing function and comprises four bar magnets at a separation so as to be located beneath the spaces between alternate rows of wells in a microtitre plate. In use, a microtitre plate is positioned over the magnetic separation station with the bar magnets aligned beneath a first set of gaps between alternate rows of wells so that magnetic beads are attracted to one side of the bottom of the wells. After a short period of time, such as 5 seconds, the plate is moved laterally the distance of the spacing between rows of wells so that the magnets are aligned beneath a second set of gaps between alternate rows of wells. In this position the magnetic beads are attracted to the opposed side of the bottom of the wells. The process is repeated a number of times, so that the magnetic beads are moved from side to side within liquid

in the wells, performing a washing function.

The base plate also supports a supply of disposable sterile pipette tips, illustrated schematically at 44, with an associated sensor 46 for determining whether a tip has been properly picked up by the robot head.

The base plate also includes a tip disposal station, illustrated schematically at 48, comprising an opening with a tip-receiving receptacle (not shown) therebelow.

The apparatus also includes regions for storage of microtitre plates awaiting processing and that have been processed.

The apparatus also includes a receptacle for temporary storage of beads which may then be recycled for further use, possibly after rejuvenation.

The apparatus further includes computer control means such as a PC (not shown) for controlling and co-ordinating movement of the robot head and associated functions in a manner that will be apparent to those skilled in the art.

In use a microtitre plate with samples to be processed is picked up by the robot head 28 and is transported between appropriate locations with suitable addition and removal of reagents to perform the desired process.

Example

The following example concerns an automated method of preparing DNA which may be carried out in the apparatus of Figure 1. The example involves an oligonucleotide probe

which has been synthesised with a biotin group at the 3' end. The biotinylated probe is attached to a streptavidin-coated magnetic bead to form a magnetic bead/probe complex. The probe is designed to be complementary to a region upstream from the M13 -21 Universal priming site.

In general, single plaques may be grown up in culture, the cells harvested and the supernatant collected and lysed to yield free single strands. The bead/probe complex is then added, and the probe allowed to anneal to the target DNA. Once bound, the bead/probe/template complex can be separated from the rest of the sample using magnetic attraction and then washed. The template may then be freed from the bead/probe complex by heating. The procedure is simple and fast and readily automated. All the post-growth steps can be carried out in microtitre plates with no centrifugation or ethanol precipitations required. In this example, 250 random M13 sub-clones were prepared and sequenced using the method outlined below.

Probe Design

The amount of template recovered depends directly on the design of the probe. The probe must be specific to the target but must not act as a secondary sequencing primer if free probe is left in solution. Consequently, the probe (shown in Figure 2) is 41bp in length and binds to a region upstream from the M13 -21 Universal primer site. The probe has a run of several 'A's at the 3' end together with the biotin group. This acts as a linker arm to prevent steric hindrance between the large streptavidin-coated beads and the binding of the probe to the target. Also the high degree of non-complementarity at the 3' end

would prevent the free probe from acting as a sequencing primer should it shear from the beads.

The probe THM13.3 was synthesised on an ABI 380B DNA synthesiser on a luMole scale. Biotin phosphoramidite was obtained from Amersham UK. The sequence of the probe was:
5' TAT CGG CCT CAG GAA GAT CGC ACT CCA GCC AGC AAA AAA Biotin A 3' (Seq. ID No. 1) Following cleavage from the column, the oligonucleotide was deprotected in ammonia at 55°C overnight. A NAP-10 column was used to purify the crude oligonucleotide.

Probe/streptavidin bead linkage

Promega nucleotide quality beads were used in this example. 1.2ml of Promega beads are used per 12 samples. The beads were washed in 0.1M NaCl three times using a neodymium-iron-boron permanent magnet to separate the beads from the washing solution. 200ul 0.1M NaCl and 10nmol THM13.3 oligonucleotide were then added to 1.2ml (1.2mg) dry beads and incubated at room temperature for 10 minutes. The beads were then washed 10 times in 0.1M NaCl to remove unbound oligonucleotide. Bead/probe complex was finally taken up in 1.2ml water. Beads may be bound to probe in bulk and stored in storage buffer at 4°C.

M13 Sub-clones

Random M13 sub-clones with 1-2kb inserts were grown up in 2ml 2xTy medium (16g bacto tryptone, 10g yeast extract, 5g NaCl in 1l water) for 5 hours at 37°C. Cells were spun down at 14,000g for 5 minutes and the supernatant transferred to microtitre plates. 400ul supernatant was

used from each sample, using two wells, each containing 200ul supernatant (Falcon 3911 MicroTest Flexible Assay Plate.)

Lysis

Prior to addition of supernatant, 10ul of 15% SDS was added to each well using an Eppendorf multidispensing pipette. Phage were lysed by heating the microtitre plate to 70°C for 10 minutes on a Techne PHC-3 cycler.

Annealing

20ul of hybridisation buffer (20% PEG 8000/2.5M NaCl) was added to each well with a multidispensing pipette; no mixing is required so the same tip may be used for all wells. Also, 20ul bead/probe complex were added to each well, and the dish was incubated at 45°C for 30 minutes.

Wash Steps

The microtitre plate was removed from the cycler and placed on a Dynal MPC-96 magnetic separator. After 30 seconds the supernatant was aspirated, 100ul wash buffer (0.1X SSC) were added to each well and the beads were moved through the wash buffer by repositioning the plate over the magnetic separator three times at intervals of five seconds. This step was repeated a total of three times. Finally, the beads were eluted in 10ul water; using the magnet, beads may be dispersed into this small volume.

Denaturation

The templates were released from the probe by heating the plate to 80°C for 3 minutes. After heating, the beads were concentrated using the magnet and the supernatant was removed to a fresh microtitre plate. Due to evaporation the final total volume was approximately 8ul from each well, or approximately 16ul per DNA template sample.

The efficacy of the technique is illustrated by Figure 3. Four random M13 subclones were grown as described. The cells were harvested and the supernatant (1200ul) was split three ways to provide three identical samples, A, B and C (400ul each). For each sub clone, Sample A was prepared using the method outlined previously. Sample B was prepared as outlined but leaving out the hybridisation buffer (20% PEG/2.5M NaCl). Sample C was prepared as outlined but using magnetic beads without a probe attached. The samples were then subjected to agarose gel electrophoresis. This showed that when the PEG is removed there is a dramatic reduction in yield, as expected. When beads are used without probe attached the resulting yield is zero. The difference between this control and the method of Alderton et al., (1992), is that in sample C the phage are lysed before addition of PEG.

Sequencing Results

Once the templates have been purified, as described above, it is possible to immediately perform the sequencing reactions as pre-reaction mixes can be made up and dispensed in advance. The ABI Taq dye primer kit reagents were used. The total volume after recovery of the template from the probe varies from 14-16ul. From this, 2ul has been used in the A/C reactions and 4ul in the G/T reactions. The volume variability does not reduce the

quality of the sequence data. All samples were sequenced using the M13 -21 universal primer and Taq polymerase. The reactions were cycled on a Techne PHC-3 dry cycler, each sample being covered with mineral oil. The reaction products were analysed on an ABI 373A DNA sequencer.

In this study, 250 clones taken from the C. elegans cosmids ZK507, ZK512 and KO1B6 were prepared using the magnetic probe method and sequenced as described above. The method gave reproducible high quality data which was assembled into current databases. Figure 4 shows a section of a read. The trace shows bases 125-375 from a random M13 sub-clone containing a 1-2kb insert. From the sequence data available, it is clear that the procedure is reproducible and not sensitive to fluctuations in template or bead concentrations. Few sub-clones failed to give sequence and overall the results were comparable to the standard PEG/phenol method.

Re-use of Beads

After the preparation of a batch of DNA samples, beads may be re-used as outlined below.

Beads were collected and pooled in a 1.5ml eppendorf tube. The supernatant was removed and the beads were resuspended in 1ml reuse buffer (0.15M NaOH, 0.001% Tween 20) for 1 minute. The supernatant was removed and the procedure repeated. Finally the beads were washed once in storage buffer and taken up in half the original volume of storage buffer (PBS pH 7.5, 0.1% BSA). Beads were then stored at 4°C.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Automated Preparation of Nucleic Acids

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TATCGGCCTC AGGAAGATCG CACTCCAGCC AGCAAAAAAA 40

Claims

1. Apparatus for preparing a DNA sequence of interest from a complex sample comprising use of magnetic particles in an assay plate, said apparatus comprising: means for moving an assay plate in three-dimensional space; means for supplying liquids to the assay plate; thermal regulation means for controlling the temperature of the assay plate; magnetic separation means for manipulating magnetic particles in the assay plate; and computer control means for controlling and co-ordinating operation of all the above means.

2. Apparatus according to claim 1 comprising an X, Y, Z robotic head.

3. Apparatus according to claim 1 or 2, comprising a robotic head with position sensing means.

4. Apparatus according to any one of claims 1, 2 or 3, comprising a robotic head capable of pipetting and dispensing liquids.

5. Apparatus according to any one of the preceding claims, wherein liquids are supplied to the assay plate by a plurality of disposable pipette tips.

6. Apparatus according to any one of the preceding claims, comprising pipette tip-sensing means.

7. Apparatus according to any one of the preceding claims, wherein the magnetic separation means comprises one or more magnetic workstations.

8. Apparatus according to any one of the preceding claims, wherein the thermal regulation means comprises three heating blocks, each set at a different temperature.

9. Apparatus according to any one of the preceding claims, comprising a static baseplate.

10. Apparatus according to any one of the preceding claims where the computer control means comprises a personal computer.

11. An automated method of preparing a DNA sequence of interest from a complex sample, comprising: use of automated means for the steps of causing lysis of DNA-containing entities by the action of heat in the presence of a suitable detergent; adding magnetic particles attached to a probe together with hybridisation buffer containing a polymer to cause an increase in the effective nucleic acid concentration; allowing the sequence of interest to hybridise to the complementary probe and then separating the particle/probe complex and any sequences hybridised thereto from the rest of the sample by magnetic attraction.

12. A method according to clam 11, comprising the use of apparatus according to any one of claims 1-10.

13. A method according to claim 11 or 12, wherein the detergent is SDS.

14. A method according to any one of claims 11, 12 or 13, wherein the DNA-contaning entities are phages.

15. A method according to any one of claims 11-14, wherein the polymer is polyethylene glycol.

16. A method according to any one of claims 11-15, performed in a microtitre assay plate.

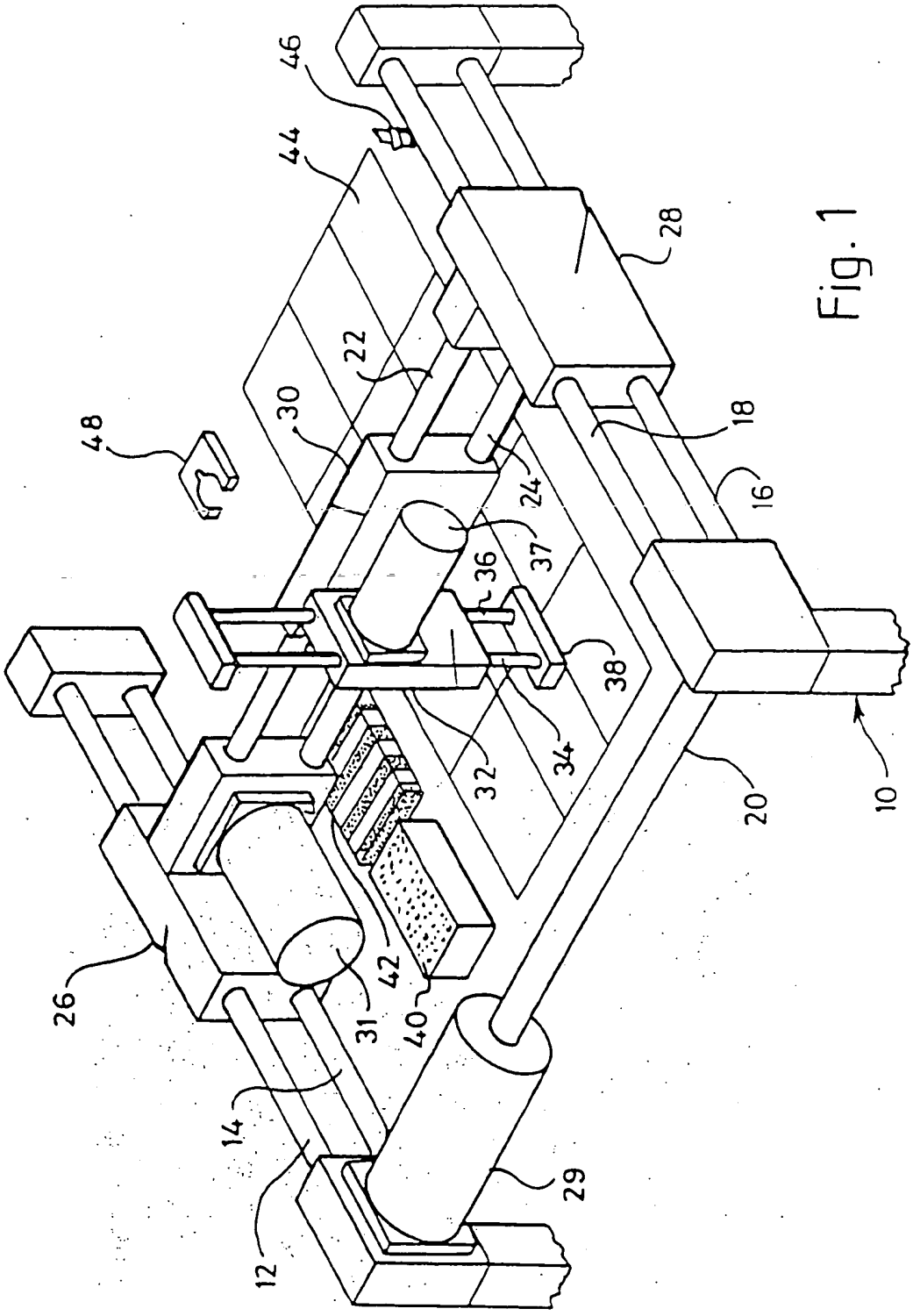
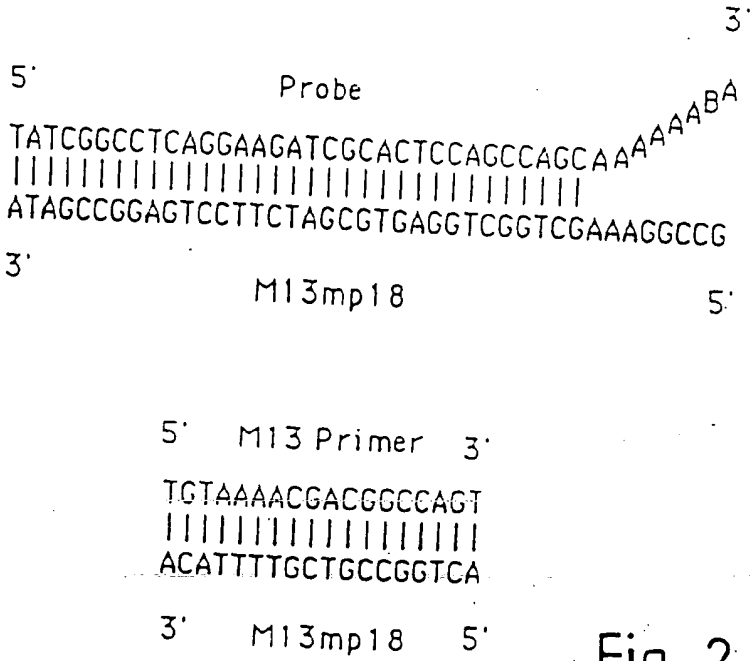


Fig. 1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Probe
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5'
5' M13 Primer 3'
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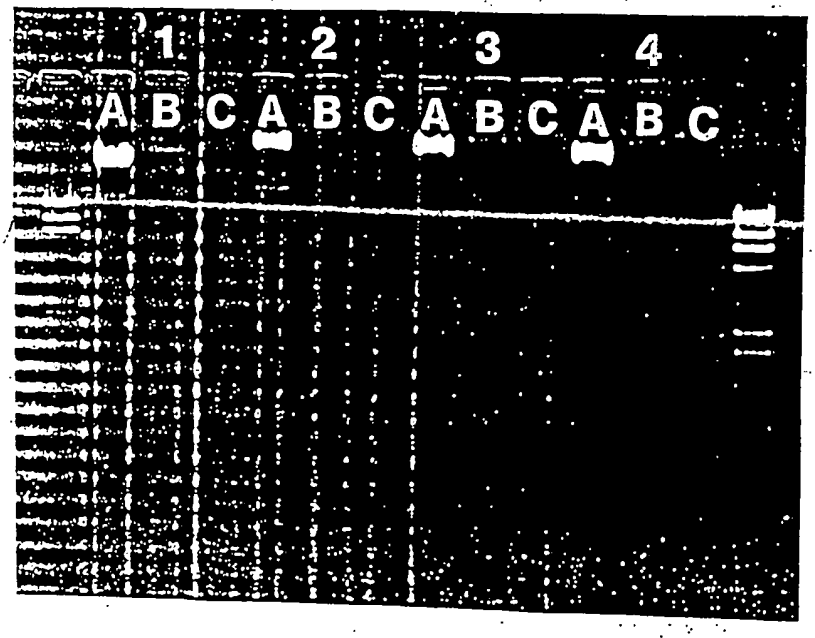
3' M13mp18 5'

Fig. 2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2
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A B C A B C A B C A B C

Fig. 3

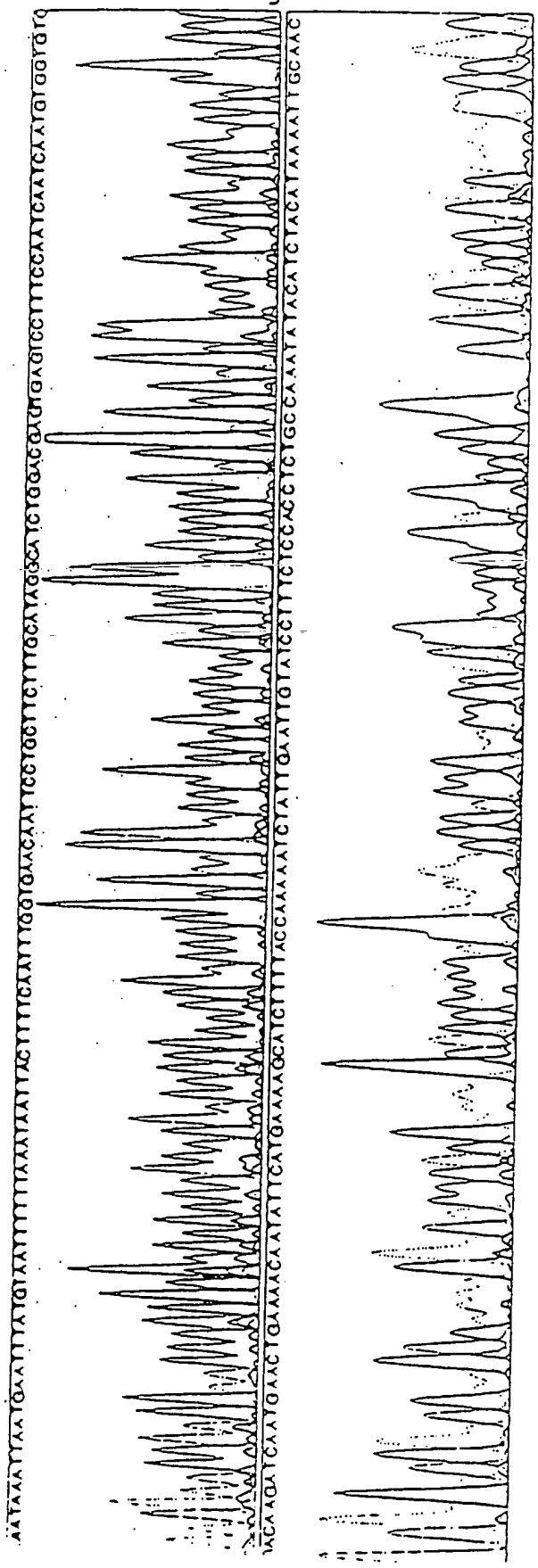

Fig. 4